SYNOPSIS

Understanding the Heat Shock Response pathway in *Plasmodium falciparum* and Identification of a novel exported Heat Shock Protein

Infections or diseases are not just stressful for the one who encounters it. The pathogens causing the same also have to deal with the hostile environment present in the host. The maintenance of physiological homeostatic balance is must for survival of all organisms. This becomes a challenging task for the protozoan parasites which often alternate between two different hosts during their life cycle and thereby encounter several environmental insults which they need to acclimatize against, in order to establish a productive infection. Since their discovery as proteins up-regulated upon heat shock, heat shock proteins have emerged as main mediators of cellular stress responses and are now also known to chaperone normal cellular functions. Parasites like *Plasmodium falciparum* have fully utilized the potential of these molecular chaperones. This is evident from the fact that parasite has dedicated about 2% of its genome for this purpose.

During transmission from the insect vector to humans, the malaria parasite *Plasmodium falciparum* experiences a temperature rise of about 10^{o}C, and the febrile episodes associated with asexual cycle further add to the heat shock which the parasite has to bear with. The exact mechanism by which the parasite responds to temperature stress remains unclear; however, the induction of chaperones such as PfHsp90 and PfHsp70 has been reported earlier. In other eukaryotes, there are three main factors which regulate heat shock response (HSR): heat shock factor (HSF), heat shock element (HSE) and HSF binding protein (HSBP). Bioinformatics analysis revealed presence of HSE and HSBP in *P. falciparum* genome; however, no obvious homolog of HSF could be identified. Either the HSF homologue in *P. falciparum* is highly divergent or the parasite has evolved alternate means to tackle

temperature stress. Therefore, we decided to biochemically characterize HSBP and understand the heat shock response pathway in the parasite using transcriptomics and proteomics. The expression for PfHSBP was confirmed at both mRNA and protein level and it was found to translocate into the nucleus during heat shock. As previously reported for HSBP in other organisms, PfHSBP also exists predominantly in trimeric and hexameric form and it interacts with PfHsp70-1. Nearly 900 genes, which represent almost 17% of the parasite genome, were found to have HSE in their promoter region. HSE are represented by three repeating units of nGAAn pentamer and its inverted repeat nCTTn; however, the most abundant class of genes in *P. falciparum* possessed an atypical HSE which had only 2 continuous repeat units. Next, we were interested to find out if these HSE could actually bind to any parasite protein. Therefore, we performed EMSA analysis with the parasite nuclear extracts using HSE sequence as the oligonucleotide. We observed retarded mobility of the oligonucleotide suggesting that it was indeed able to recruit some protein from the nuclear extract. The importance of transcriptional regulation during heat shock was further confirmed when parasite culture subjected to heat shock in the presence of transcription inhibitor did not show induction in the levels of PfHsp70. These evidences suggest that parasite indeed possesses all the components of heat shock response pathway with either a divergent homologue of HSF or an alternate transcription factor which would have taken its role. Next, we performed global profiling of heat shock response using transcriptomic analysis and 2D-DIGE based proteomic profiling. Overall, the parasite's response to heat shock can be classified under 5 functional categories which aim at increasing the folding capacity of the cell, prevent protein aggregation, increase cytoadhesion, increase host cell remodelling and increase erythrocyte membrane rigidity. Out of the 201 genes found to be up-regulated upon heat shock, 36 were found to have HSE in their promoter region. This suggested that HSE-mediated protein up-regulation could be responsible for the induction of only 18% of total

number of genes up-regulated upon heat shock. How would the parasite bring about up-regulation of rest of the heat shock responsive genes? It has been previously reported that genes for some of the heat shock proteins in *P. falciparum* possess G-box regulatory elements in their promoters and recently, it was shown that these elements served as the binding site for one of the transcription factors (PF13_0235) of AP2 family. Therefore, we looked for the status of this AP2 factor and its targets in our transcriptome data. Although, PF13_0235 was itself not up-regulated, we found up-regulation of its target genes which included another AP2 factor gene PF11_0404. The target genes of PF11_0404 were also up-regulated upon heat shock, thereby suggesting the functioning of an AP2 factor mediated response to heat shock.

The next major challenge which the malaria parasite has to deal with is the remodelling of the erythrocyte as these cells do not have a cellular machinery which the parasite can take control of. The parasite remodels the erythrocyte with the help of its large repertoire of exported proteins and develops protrusions known as “knobs” on the erythrocyte surface. These protrusions are cytoadherent in nature and constitute the main virulence determinants of malaria. They also represent variable antigens that allow immune escape. Our lab has previously demonstrated an exported PfHsp40, termed as KAHsp40, to be involved in knob biogenesis. Apart from KAHsp40, there are 19 other PfHsp40s which possess the PEXEL motif required for protein export to erythrocytes. Although, Hsp40s work with an Hsp70 partner, none of the parasitic Hsp70s were known to be exported and was always a missing link in the field of malaria chaperone biology. A genomic re-annotation event could fill this gap by re-annotating the sequence for a pseudogene, PfHsp70-x and described it to contain a functional ORF. According to the re-annotated ORF sequence, PfHsp70-x possessed an ER signal peptide and thus could be targeted to the secretory pathway.

Following validation of the re-annotation using a PCR-based approach, we confirmed the expression of this protein at the protein level by immunoblot analysis. Using various sub-cellular fractionation approaches and immunolocalization studies we established that PfHsp70-x indeed gets exported to the erythrocyte compartment; however, it did not contain the PEXEL motif required for protein export. It gets secreted into the vacuole around the parasite via the canonical ER-Golgi secretory pathway. Its trafficking from vacuole into the erythrocyte was mediated by a hexameric sequence which was present just after the signal peptide cleavage site and before the beginning of ATP-binding domain. In the erythrocyte compartment, it was found to interact with KAHsp40 and MAHRP1, proteins previously implicated in knob biogenesis. Most importantly, PfHsp70-x interacted with the major knob component PfEMP1; however, itself did not become part of knobs. Instead, it localized to the Maurer's clefts in the erythrocyte compartment. Inside the parasite, PfHsp70-x was present in a complex with Plasmepsin V and PfHsp101. These proteins have been shown to be essential for host cell remodelling process. Plasmepsin V recognizes the PEXEL motif and brings about its cleavage and PfHsp101 specifically targets these PEXEL-cleaved exported proteins to the translocon in vacuolar membrane thereby facilitating their export into the erythrocyte. Thus, PfHsp70-x could also be involved in directing the export of knob constituents apart from just facilitating their assembly. Since, we found out that heat shock or the febrile episodes encountered during the asexual cycling of the parasite promote host cell remodelling; we wanted to find out if PfHsp70-x has any specific role under conditions of temperature stress. PfHsp70-x gene expression was not influenced upon heat shock, however, its export into the erythrocyte was inhibited and the protein got accumulated within the parasite compartment. Surprisingly, immunolocalization studies revealed that the accumulated pool of PfHsp70-x localized into the nucleus instead of ER thus suggesting an alternate role to be associated with PfHsp70-x under stress.

Overall, our study addresses two major aspects of malaria pathogenesis. First, response to heat shock and second, remodelling of the host cell. We, for the first time describe global profiling of the parasite's heat shock response and identify a novel *P. falciparum* specific heat shock protein member to be involved in malaria pathogenesis.